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Patent Application
Docket No. UF-214XC1
Serial No. 09/201,568

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Eliane Lazar-Wesley, Ph.D.
Art Unit	:	1646
Applicants	:	Dov Borovsky
Serial No.	:	09/201,568
Filed	:	November 30, 1998
For	:	TMOF Receptor and Uses Thereof

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF DOV BOROVSKY UNDER 37 CFR §1.132

Sir:

I, Dov Borovsky, Ph.D., of the University of Florida, Florida Medical Entomology Laboratory, hereby declare:

THAT, I am a named inventor on the above-referenced patent application;

THAT, I have received the following degrees:

Ph.D. Biochemistry 1972 University of Miami, Miami FL

B.A. Bacteriology 1967 University of California, Los Angeles, CA;

THAT, I have been employed professionally as follows:

1999 - present Visiting Professor of Insect Biochemistry and Molecular Biology, Katholieke Universiteit, Zoology Institute, Leuven

1991 - present Adjunct Professor, University of Miami, School of Medicine, Department of Biochemistry and Molecular Biology, Miami FL

1985 - 1991 Adjunct Associate Professor, University of Miami, School of Medicine, Department of Biochemistry and Molecular Biology, Miami FL

1990 - present Professor of Insect Biochemistry, Institute of Food and Agricultural Sciences, University of Florida, Florida Medical Entomology Laboratory, Vero Beach, FL

1983 - 1990 Associate Professor of Insect Biochemistry, Institute of Food and Agricultural Sciences, University of Florida, Florida Medical Entomology Laboratory, Vero Beach, FL

1978 - 1983 Research Biochemist, Florida Medical Entomology Laboratory, Vero Beach, FL

1975 - 1978 Chemist III, Florida Medical Entomology Laboratory, Vero Beach, FL

1975 Research Assistant Professor, University of Miami, School of Medicine, Department of Biochemistry, Miami, FL

1973 - 1975 Research Instructor, University of Miami, School of Medicine, Department of Biochemistry, Miami, FL

1972 - 1973 Post Graduate Research Biochemist, University of California - Davis, School of Medicine, Department of Biochemistry, Davis, CA

1967 - 1972 Teaching Assistant, University of Miami, School of Medicine, Department of Biochemistry, Miami, FL;

THAT, I have authored approximately 111 Publications (1972-2000), including 2 books, 18 book contributions, 63 publications in refereed journals, and 28 publications in other journals;

THAT, I am a named inventor on 3 U.S. Patents, including U.S. Patent No. 5,358,934, "Materials And Methods For Control of Pests", issued October 25, 1994; U.S. Patent No. 5,130,253, "DNAs Encoding Mosquito Oostatic Hormones", issued June 14, 1992; and U.S. Patent No. 5,011,909, "Novel Compositions And Process For Inhibiting Digestion In Blood-Sucking Insects", issued April 30, 1991;

THAT, through my years of research, I have kept up to date on the technical literature and maintained contact with experts in the field by participating in professional meetings and seminars, and by direct personal contact. As a result, I am familiar with the general level of skill of those working in the fields of entomology and microbiology, and in particular the field of biocontrol of pests;

THAT, I have read and understood the specification and claims of the subject application and the Office Action dated April 11, 2001;

AND, being thus duly qualified, do further declare:

1. Trypsin Modulating Oostatic Factor (TMOF) is a hormone common to mosquitoes and many other pests, including, among others, fleshflies, fleas, sand flies, house flies, and dogflies, that digest their food using trypsin and trypsin-like enzymes. TMOF's role in the digestive process is to inhibit digestive enzyme biosynthesis by binding to specific receptors. These receptors are present in all mosquitoes and other insects that produce TMOF hormone, thereby permitting such receptors to be harvested and characterized from a multitude of different species.

2. It is well known in the art that TMOF receptors across a wide spectrum of insects, such as *Aedes aegypti*, *Culex quinquefasciatus*, *Anopheles albimanes*, *Auopheles quadrimaculates*, *Lutzomyia anthrophora*, *Culicoides variipennis*, *Stomoxys calcitrans*, *Musca domestica*, and *Ctenocephalides felis* show an affinity to bind TMOF-like enzymes, which exhibit a high degree of homology. See U.S. Patent No. 5,130,253. As a result, research which pertains to the TMOF hormone, its analogs, and TMOF receptors of one species of pest is applicable to other species of pest which utilize this pathway in their digestive process. Therefore, although the experiments which led to the subject invention were performed using a particular species of mosquito (*Aedes aegypti*), as indicated at page 2, lines 8-10, of the subject application, those skilled in the art can,

using the teachings provided in our patent application, readily obtain and use TMOF receptors, and polynucleotides encoding these receptors, from other species.

3. The following is a brief background of the complementary peptide approach, which was utilized to arrive at the subject invention. In a paper published in 1985 in PNAS USA, Bost *et al.* reported the binding of peptides (termed complementary peptides) that are encoded by nucleotide sequences that are complementary to each other (Bost *et al.*, "Similarity Between the Coritcotropin (ACTH) Receptor and a Peptide Encoded by an RNA that is Complementary to ACTH mRNA," *Proc. Natl. Acad. Sci. USA* 82, 1372-1375 (1985)). Using this concept, it has been found that many peptides complementary to hormones, including corticotropin (ACTH), γ-endorphin, or luteinizing hormone-releasing hormone (LHRH), bind the appropriate hormone and that the immune system recognizes these peptides as antigenically similar to the respective hormone receptor binding site (Jarpe, M.A., and Blalock, J.E. "Complementary Peptides: Applications of the Molecular Recognition Theory to Peptide and Protein Purification and Design," *Peptides: Design, Synthesis, and Biological Activity* (Busava and Ananthramaiah, eds)).

4. In developing the subject invention, the sequence of the complementary peptide for TMOF hormone ("FOMT") was determined by the methods outlined in the above referenced chapter in *Neuroendocrine Peptide Methodology*, by Bost and Blalock (Bost, K.L. and Blalock, J.E. (1989) "Preparation and Use of Complementary Peptides" *Neuroendocrine Peptide Methodology*, 697-709), the substance of which is incorporated herein by reference.

5. After synthesizing the TMOF complementary peptide (FOMT), it was determined that the complementary peptide successfully competes with the TMOF gut receptor, and specifically binds with the TMOF hormone (Borovsky, D. *et al.* (1994) "Characterization

and Localization of Mosquito-gut Receptors for Trypsin modulating Oostatic Factor Using a Complementary Peptide and Immunohistochemistry" *FASEB J.* 8:350-355).

6. Once I determined that the complementary peptide (FOMT) behaves as a synthetic receptor in effectively binding TMOF, I synthesized a degenerate oligonucleotide sequence based on the amino acid sequence of the complementary peptide (FOMT). The design and use of degenerate oligonucleotides is also well known in the art. (*See* "Consideration and Design of Primers" (1998) *Molecular Biomethods Handbook* (Rapley, R. and Walker, J.M., eds.), 307-308).

7. Since every mRNA has a polyA tail at the 3' end, I then performed a high stringency 3' Rapid Amplification of cDNA Ends (RACE) polymerase chain reaction (PCR) procedure, utilizing the polyA tail as a priming site and the degenerate oligonucleotide on the 5' end. Using the 3' RACE procedure, which is also well known in the art, I successfully isolated the 378 base pair cDNA sequence (including the polyA end) from a cDNA library of the mosquito, *Aedes aegypti*. This cDNA sequence is set forth as SEQ ID NO. 1 in the patent application, and encodes the amino acid sequence set forth as SEQ ID NO. 2.

8. The methods outlined in the above paragraphs do not represent the only methods of isolating the receptor of the subject invention and are provided herein as evidence of the claimed receptor's utility, *e.g.*, TMOF binding capability. Therefore, I submit that one skilled in the art would appreciate the functional characteristics of the subject invention; this would be based upon the knowledge gleaned from relevant publications in the art, such as those referenced above, and the teachings of the patent application itself.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisionment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued therefrom.

By: _____ Date: 7/6/01
Dov Borovsky

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Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1372–1375, March 1985
Biochemistry

Similarity between the corticotropin (ACTH) receptor and a peptide encoded by an RNA that is complementary to ACTH mRNA

(genetic code/receptor–hormone binding/evolution/peptide–peptide interaction)

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Communicated by George K. Davis, October 25, 1984

ABSTRACT **An interesting pattern in the genetic code was recently observed: Codons for hydrophilic and hydrophobic amino acids on one strand of nucleic acid are complemented by codons for hydrophobic and hydrophilic amino acids on the other strand, respectively. The average tendency of codons for "uncharged" (slightly hydrophilic) amino acids is to be complemented by codons for "uncharged" (slightly hydrophilic) amino acids. We have postulated that this pattern can result in the binding of peptides that are encoded by complementary RNA strands and we have presented supporting evidence. In this report we demonstrate the specific and high-affinity binding of naturally occurring peptides [corticotropin (ACTH) and γ-endorphin] to synthetically derived counterparts that were specified by RNA sequences complementary to the mRNA for ACTH and γ-endorphin, respectively. That this binding might result from one peptide being an "internal image" of the other was strongly suggested by the observation that antibody to the peptide that was encoded by the complementary RNA for ACTH recognized the adrenal cell ACTH receptor. Based on these findings, a theory on the evolution of peptides and their receptors is suggested.**

We have recently observed that codons for hydrophobic amino acids are complemented by those for hydrophilic amino acids and vice versa. The average tendency of codons for "uncharged" (slightly hydrophilic) amino acids is to be complemented by codons for "uncharged" (slightly hydrophilic) amino acids (1). Following this pattern, two peptides that represent complementary strands of nucleic acid would display an interchange of their hydrophilic and hydrophobic amino acid residues when the amino terminus of one is aligned with the carboxyl terminus of the other.

One possible consequence of the aforementioned observation is suggested by the finding that many biologically important peptides composed of 10 to 50 amino acids, such as hormones, can assume amphiphilic secondary structures in the presence of another amphiphilic structure such as a membrane or a receptor binding site (for review see ref. 2). One face of the peptide is thought to be preferentially occupied by lipophilic side chains, whereas all hydrophilic residues are located at the opposite face. In fact, in the presence of their receptors, peptide hormones have been predicted to have well-defined amphiphilic conformations (3, 4). For hormone–receptor interaction and binding to occur, the two structures must undoubtedly be complementary with respect to their hydrophobic and hydrophilic domains. Thus we theorized that two peptides that are encoded by complementary nucleic acid strands might form or impose such amphiphilic structures and bind one another.

To test this idea, peptides encoded by RNA sequences complementary to the mRNA of corticotropin (ACTH) residues 1–24 and γ-endorphin (5) were synthesized and assayed for their ability to bind ACTH and γ-endorphin, respectively. In this report, we demonstrate the specific and high-affinity binding of such peptide pairs. The possible biologic or evolutionary significance of this finding is suggested by the further observation that an antibody to the peptide encoded by the complementary RNA for ACTH seems to recognize the ACTH receptor. Based on these findings, a theory on the evolution of peptides and their receptors is suggested.

MATERIALS AND METHODS

Peptides. Peptides were synthesized according to the RNA sequences complementary to the mRNAs of bovine ACTH-(1–24) and bovine γ-endorphin (5) by Peninsula Laboratories (San Carlos, CA). The final products were estimated to be 91% peptide and 9% very short fragments and free amino acids. Synthetic ACTH-(1–24) and synthetic γ-endorphin were obtained from Organon and Boehringer Mannheim, respectively.

Antisera. Rabbit antisera to synthetic ACTH-(1–13) amide and synthetic γ-endorphin were obtained from Accurate Biochemicals (Westbury, NY). A rabbit antiserum (abbreviated anti-HTCA) to the synthetic peptide that corresponded to the complementary RNA sequence of ACTH-(1–24) was prepared in this laboratory. Peptide (200 μg) was coupled to keyhole limpet hemocyanin (KLH; 200 μg) with glutaraldehyde (6.7 mM) (6). Excess glutaraldehyde was removed by passage through a Bio-Rad P-10 column. Three injections (25 μg each) of the peptide-KLH conjugate in 0.5 ml of complete Freund's adjuvant were administered at 2-week intervals. Total immunoglobulin from the resulting antiserum was purified on an affinity column of Sepharose 4B-coupled goat antibody to rabbit immunoglobulin. Antibodies to KLH were removed from the purified immunoglobulin by passage over a KLH-Sepharose 4B affinity column. A 1:300 dilution of this antibody would detect at least 100 ng of the complementary peptide for ACTH in an indirect enzyme-linked immunosorbent assay (ELISA).

ELISA. The indicated amounts of complementary strand peptides, insulin, or bovine serum albumin in carbonate/bicarbonate coating buffer were added to 96-well round-bottom microtiter plates and incubated at 4°C for 18 hr as previously described (7). Plates were washed three times with phosphate-buffered saline/Tween 20 and either ACTH or γ-endorphin in phosphate-buffered saline/Tween was added (amounts are indicated in individual figure legends). Controls received an equivalent volume of buffer. Plates were incubated for 1 hr at room temperature and then washed three times with phosphate-buffered saline/Tween. Any ACTH or γ-endorphin that bound to the previously coated plates was then detected with rabbit antisera to synthetic

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Abbreviations: ACTH, corticotropin; HTCA, a peptide synthesized according to the RNA sequence complementary to the mRNA of bovine ACTH-(1–24); KLH, keyhole limpet hemocyanin.

*To whom reprint requests should be addressed.

ACTH and γ-endorphin, respectively. Briefly, the primary antiserum in phosphate-buffered saline/Tween was added and the plates were incubated for 1 hr at room temperature. After three washes with phosphate-buffered saline/Tween, alkaline phosphatase-conjugated goat antibody to rabbit IgG in phosphate-buffered saline/Tween (1:300 dilution) (Miles) was added. After 1-hr incubation, the plates were washed three times with phosphate-buffered saline/Tween and the complex was allowed to react with 200 μl of *p*-nitrophenyl phosphate (1 mg/ml in carbonate/bicarbonate buffer) for 1.5 hr at room temperature. The reaction was stopped with 50 μl of 3 M NaOH and the absorbance of the fluid in each well was measured at 405 nm.

ELISAs were also performed on glutaraldehyde-fixed mouse adrenal tumor (Y-1) cells in flat-bottomed microtiter plates. In these assays, rabbit antibody to the peptide encoded by the complementary RNA for ACTH-(1–24) was the primary antibody. The secondary antiserum was added and the ELISA was then performed as previously described.

ACTH Bioassay. Mouse adrenal tumor (Y-1) cells in microtiter plates were treated with either ACTH or antibody to the peptide encoded by the complementary RNA for ACTH-(1–24) mRNA, which was previously diluted in F-10 medium containing 15% horse serum and 2.5% fetal calf serum. After a 16-hr incubation at 37°C in a 4% CO_2 atmosphere the cell culture supernatant fluids were assayed for glucocorticoid hormone production in a previously described radioimmunoassay for corticosterone (8).

Purification of the ACTH Receptor. Purified antibody (anti-HTCA) to the peptide encoded by the RNA sequence complementary to the mRNA of ACTH-(1–24) was covalently coupled to CNBr-activated Sepharose 4B as previously described. Approximately 1×10^8 Y-1 cells were sonicated for 5 min at 40 kHz (Branson E Module bath sonicator) in the presence of 2 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation and the supernatant fluids were passed over the anti-HTCA affinity column. The column was extensively washed and then eluted with 0.1 M glycine (pH 2). The eluted material was restored to neutral pH and placed in a dialysis bag for hydroextraction with polyethylene glycol. Concentrated material was then filtered through a Sephacryl S-200 column (Pharmacia). Samples from the gel filtration column were assayed for ACTH receptor activity by a radioreceptor procedure. Briefly, aliquots of the gel filtration fractions were incubated in 96-well polyvinyl plates for 18 hr and the nonbound material was removed. ^{125}I-labeled ACTH (^{125}I-ACTH) (70 μCi/μg, New England Nuclear; 1 Ci = 37 GBq) was then added in the presence or absence of an excess of unlabeled ACTH (10 μg per well). Plates were then extensively washed, wells were cut out of the plates, and their radioactivities were measured in a Beckman Gamma 5500 γ counter. Specifically bound radioactive material is total cpm bound minus those bound in the presence of excess unlabeled ACTH. In those fractions that bound ^{125}I-ACTH, greater than 90% of the cpm was specifically bound.

RESULTS

Specific Binding of Peptides Encoded by Complementary RNAs. Initially, a peptide (HTCA) corresponding to the complementary RNA sequence of ACTH-(1–24) mRNA (5) was synthesized (Fig. 1) and assayed for the ability to bind ACTH. Fig. 2 shows that HTCA is capable of binding synthetic ACTH as determined by an ELISA with monospecific antiserum to synthetic ACTH-(1–13) amide. Under identical conditions, another peptide (insulin) did not bind ACTH, and HTCA alone was not recognized by antiserum to ACTH. The molar amount of ACTH bound was directly proportional to the concentration of HTCA, which suggests a one-to-one binding of the two peptides (data not shown). To determine the amount of specific binding and the affinity of the interaction, competitive binding experiments with free versus solid-phase HTCA were performed. Fig. 3 shows that 90% of the binding of ACTH to HTCA was specific. A Scatchard analysis of these data showed a single uniform binding site with a K_d of 1.9 μM. This affinity is on the order of 5×10^7 M^{-1}, which is comparable with the affinity of 1×10^8 M^{-1} previously reported for the low-affinity ACTH receptor of the rat (9). Taken together, these data show that the interaction between ACTH and HTCA is specific and exhibits high affinity.

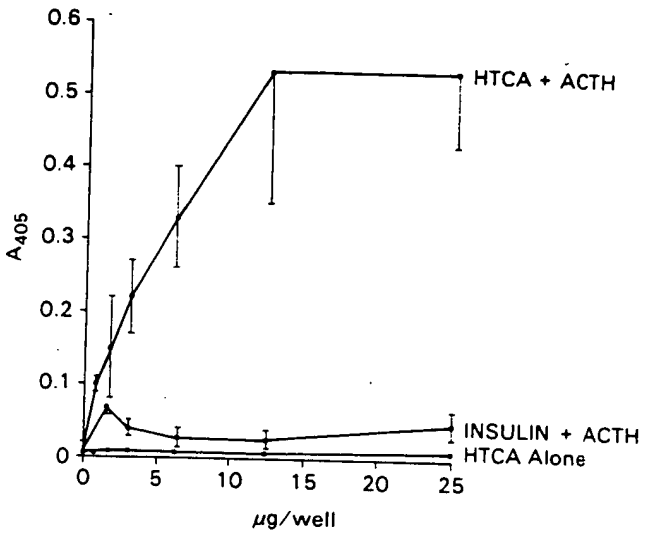

FIG. 2. Binding of ACTH by HTCA. The complementary peptide, HTCA, or insulin was coated onto microtiter wells in the indicated amounts. Synthetic ACTH-(1–24) in phosphate-buffered saline/Tween was then added at 10 μg per well. Plates were incubated for 2 hr and then washed three times with phosphate-buffered saline/Tween. The amount of ACTH bound was determined by an ELISA using monospecific antisera to synthetic ACTH-(1–13) amide. Control wells (HTCA alone) were incubated with phosphate-buffered saline/Tween instead of ACTH.

Although the likelihood of randomly choosing a 24 amino acid sequence that behaves in the above fashion seemed extremely remote, a second peptide combination was tested to ensure that we were not dealing with a chance occurrence. This second peptide corresponded to the complementary RNA for bovine γ-endorphin mRNA (5) and had the following sequence: Gln-Arg-Asp-Lys-Gly-Arg-Leu-Ala-Leu-Leu-Gly-Gly-His-Glu-Pro-Ala-Val. Fig. 4 shows that this peptide is capable of binding synthetic γ-endorphin as determined by an ELISA with monospecific antiserum to γ-endorphin. Under identical conditions, another peptide (insulin) and a pro-

ACTH		Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val	Gly	Lys	Lys	Arg	Arg	Pro	Val	Lys	Val	Tyr	Pro	
+RNA	5'-UCU	UAC	UCC	AUG	GAA	CAC	UUC	CGC	UGG	GGC	AAG	CCG	GUG	GGC	AAG	AAG	CGG	CGC	CCG	GUG	AAG	GUG	UAC	CCC-3'	
−RNA	5'-GGG	GUA	CAC	CUU	CAC	CGG	GCG	CCG	CUU	CUU	GCC	CAC	CGG	CUU	GCC	CCA	GCG	GAA	GUG	UUC	CAU	GGA	GUA	AGA-3'	
HTCA		Gly	Val	His	Leu	His	Arg	Ala	Pro	Leu	Leu	Ala	His	Arg	Leu	Ala	Pro	Ala	Glu	Val	Phe	His	Gly	Val	Arg	

FIG. 1. Amino acid sequence of a peptide (HTCA) encoded by RNA that is complementary to ACTH-(1–24) mRNA. The sequences +RNA (which encodes ACTH) and −RNA (which encodes HTCA) are from ref. 5.

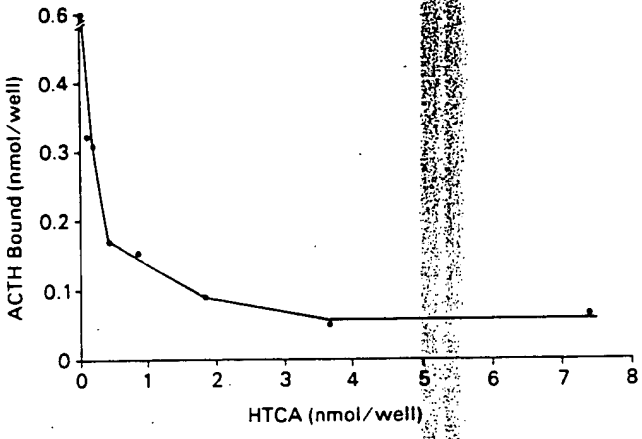

FIG. 3. Specific binding of ACTH by HTCA. The indicated concentrations of soluble HTCA were incubated with ACTH (3.7 nmol per well) prior to adding the mixture to microtiter plates containing previously bound (solid-phase) HTCA (3.7 nmol per well). The amount of ACTH binding at each concentration of soluble HTCA was determined by comparing the A_{405} with that of a standard ELISA curve for ACTH-(1–24).

tein (bovine serum albumin) did not bind γ-endorphin and the complementary peptide alone was not recognized by antiserum to γ-endorphin. When free complementary peptide was incubated with γ-endorphin, the subsequent binding to solid-phase peptide was completely blocked. Thus a second pair of peptides that are encoded by complementary RNAs specifically bind one another.

Monospecific Antibody to a Peptide That Is Specified by the Complementary RNA Sequence of ACTH mRNA Binds the ACTH Receptor. Clearly, chemical considerations preclude the binding of ACTH and HTCA through an interaction of hydrophobic regions of one peptide with hydrophilic regions of the other. Further, the two peptide sequences do not seem compatible with only a simple charge interaction. Thus, by elimination, the shape or spatial configuration of the two peptides would seem important to their binding. Since undoubtedly the adrenal cell ACTH receptor has a shape or spatial configuration that is conducive to ACTH binding, and this conformation may be reflected in the receptor's antigenicity, we determined whether HTCA is antigenically related to the ACTH receptor. Table 1 shows that monospecific antibody to HTCA caused an ACTH-like steroidogenic response on cultured mouse adrenal cells, presumably by binding to the ACTH receptor. Such activation of receptor-mediated responses by anti-receptor antibody is known to occur with other receptors, such as those for insulin and β adrenergic agents (10, 11). Normal rabbit serum and antibody to the carrier protein for immunization (KLH) did not cause a steroidogenic response (data not shown).

Table 1. Induction of steroidogenesis by antibody to HTCA

Addition	Corticosterone equivalents, μg/ml
Medium	0.67 ± 0.01
ACTH	1.25 ± 0.24*
Anti-HTCA	1.11 ± 0.11†

Duplicate cultures of mouse adrenal (Y-1) cells in microtiter plates were treated with culture medium, ACTH (10 microunits per well), or antibody to HTCA (1:3 dilution). After 18 hr of incubation at 37°C in 4% CO_2, replicate cultures were pooled and assayed for glucocorticoid hormone production by a radioimmunoassay (RIA) for corticosterone (8). Parallel dose responses for experimental samples and the corticosterone standard were obtained over a 10-fold range. Interassay variation was 8.8%. Results represent the mean ± SD of duplicate experiments.
*Different from the control at a $P \leq 0.05$.
†Different from the control at a $P \leq 0.01$.

Purified antibody to HTCA, but not the carrier protein for immunization (KLH), was directly observed to bind to glutaraldehyde-treated mouse adrenal cells by an ELISA. ACTH, in a dose-dependent fashion, blocked this binding (Fig. 5). Thus, ACTH and antibody to HTCA seemed to compete for the same adrenal cell binding site. To study the physicochemical nature of the antigenic site that was detected in the ELISA, a sonicate of mouse adrenal cells was passed over an anti-HTCA affinity column and the bound material was filtered through Sephacryl S-200. Fig. 6 shows that the material that bound to the affinity column eluted at a position that corresponded to a molecular weight of 80,000 to 130,000. Furthermore, ^{125}I-ACTH was specifically bound by those fractions corresponding to the peak. This molecular weight is similar to the 100,000 that was previously reported for the ACTH receptor on the basis of a photoaffinity labeling technique (12). Taken together, these results strongly suggest that antibody to HTCA recognized the ACTH receptor.

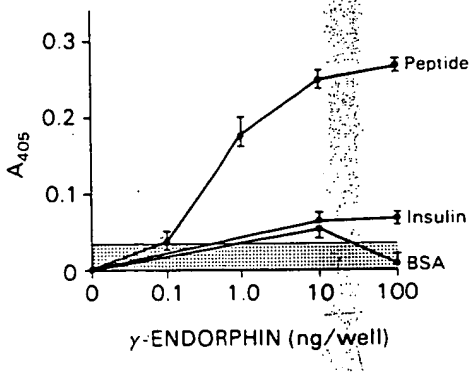

FIG. 4. γ-Endorphin binding to a peptide encoded by the complementary RNA for bovine γ-endorphin. γ-Endorphin at various concentrations was incubated on microtiter plates coated with the complementary peptide (40 μg per well), insulin (20 units per well), or bovine serum albumin (BSA; 200 μg per well). Plates were incubated for 1 hr, then washed three times with phosphate-buffered saline/Tween. The amount of γ-endorphin bound was detected by an ELISA using a monospecific antiserum to γ-endorphin. The shaded region demonstrates the amount of γ-endorphin binding to the solid-phase complementary peptide in the presence of excess soluble peptide (200 μg/ml).

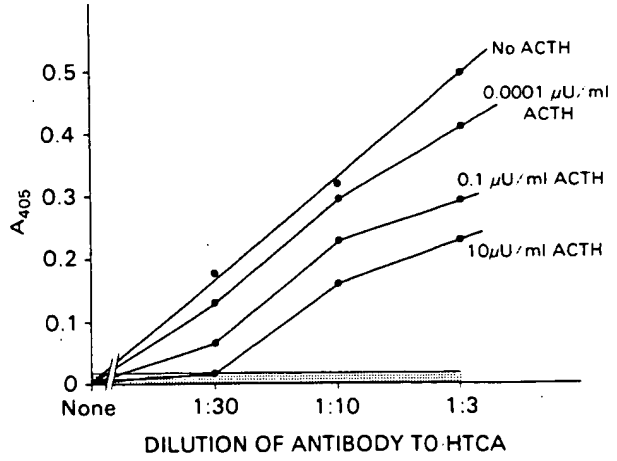

FIG. 5. Blocking of anti-HTCA binding to mouse adrenal (Y-1) cells by synthetic ACTH-(1–24). Anti-HTCA binding to glutaraldehyde-treated Y-1 cells was determined in the absence of ACTH or in the presence of synthetic ACTH-(1–24) at various concentrations. Antibody to KLH (shaded region) did not bind to Y-1 cells. U, ACTH unit.

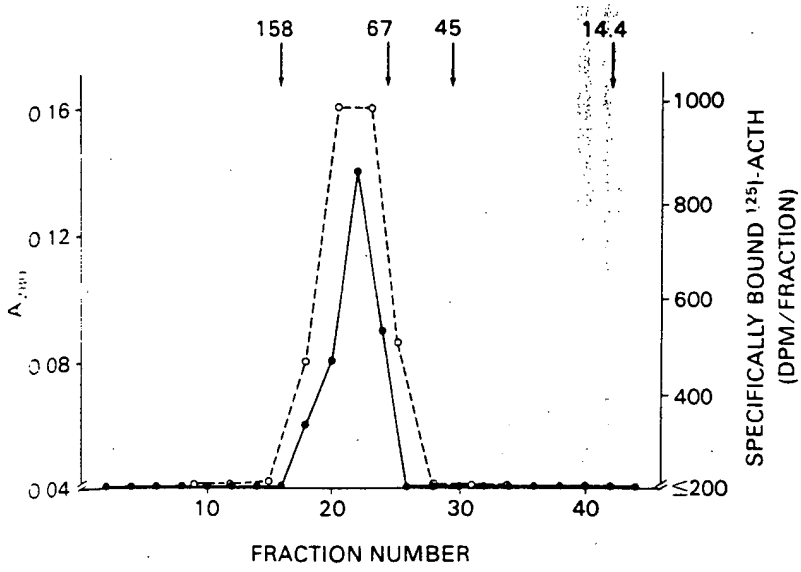

FIG. 6. Gel filtration of an antigen from Y-1 adrenal cells that specifically bound to an anti-HTCA antibody affinity column. The material from Y-1 cell sonicates that specifically bound to an anti-HTCA affinity column was filtered through Sephacryl S-200. Fractions were analyzed for absorbance at 280 nm (●) and then coated onto microtiter plates. The dpm/fraction of specifically bound ^{125}I-ACTH (○) was determined by a radioreceptor assay. Molecular weight standards eluted from the Sephacryl column as shown by the arrows (labeled as molecular weight × 10^{-3}).

DISCUSSION

An interesting pattern in the genetic code that allows for hydropathic anticomplementarity of amino acids was previously observed (1). We have theorized that a result of this relationship is that complementary DNAs, when transcribed in the 5′ to 3′ direction and in the same reading frame, will code for peptides or proteins that interact. This theory has been tested and is supported by the specific and high-affinity binding of naturally occurring peptides (ACTH and γ-endorphin) to synthetically derived counterparts that were specified by the complementary RNA sequences for ACTH and γ-endorphin mRNA, respectively. That this binding might result from one peptide being an "internal image" of the other was strongly suggested by the observation that antibody to HTCA recognized the adrenal cell ACTH receptor. Thus, the complementarity that is a hallmark of nucleic acids may result in complementarity in terms of binding of the peptides for which they code.

There would seem to be numerous implications to this theory. In a practical sense, small and well-defined peptides that interact may result. These could provide x-ray crystallographers and protein chemists with very useful tools for the study of peptide–peptide binding. Additionally, many peptide receptors have proven difficult to chemically isolate and study. Antibodies to the peptides encoded by the complementary nucleic acid strands may allow for easy purification and characterization of receptors. In a theoretical sense, these findings could provide a conceptual framework for the evolution of ordered peptide–peptide interactions that is based on the complementarity of nucleic acids. Evolutionarily, one could imagine all that might be required for the generation of a peptide and its primordial receptor was the translocation of the peptide's complementary DNA sequence. Further, this may be one reason for the usual lack of transcription in the same reading frame and in the same cell of both strands of DNA that represent a particular gene. These findings may also be important in the understanding of differentiation. For instance, the mere transcription of a DNA sequence by one cell and the complementary sequence by another could allow for cellular recognition and communication via the interacting protein products. These ideas should be testable by searching for regions of complementarity among messenger RNAs from interacting cell types such as neural, pituitary, and adrenal cells and idiotype- and anti-idiotype-bearing lymphocytes. If positive, such findings could provide a genetic and molecular basis for the concepts of internal imaging in the immune system and circuit formation in the central nervous system.

Note Added in Proof. It has come to our attention that the hydropathic anti-complementarity of amino acids described in ref. 1 for complementary codons read in the 5′-to-3′ direction also occurs in the 3′-to-5′ direction. Hence, theoretically, the RNA for HTCA may result in a peptide that binds ACTH regardless of whether amino acids are assigned in the 5′-to-3′ or 3′-to-5′ direction. Also, we have recently observed regions of complementarity between mRNAs for epidermal growth factor, transferrin, and interleukin 2 and their respective receptors.

We acknowledge the encouragement of our colleagues in the Department of Microbiology, particularly Drs. Gerald M. Fuller, G. John Stanton, and Samuel Baron, and the expert technical assistance of Tom Kruger and Audrey Morrill. We thank Dr. Walter Meyer for encouragement and the performance of corticosterone assays. We also thank Diane Weigent for typing the manuscript. K.L.B. was supported by a McLaughlin postdoctoral fellowship.

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[44] Preparation and Use of Complementary Peptides

By KENNETH L. BOST and J. EDWIN BLALOCK

Introduction

Previous experiments have shown that peptides encoded by complementary strands of nucleic acids (designated "complementary" peptides) using the same reading frame have the ability to bind one another.[1-3] Not only could peptides complementary to corticotropin (ACTH), γ-endorphin, or luteinizing hormone-releasing hormone (LHRH) bind the appropriate hormone, the immune system recognized the complementary peptides as being antigenically similar to the respective hormone receptor binding site.[1,4-7] Stated differently, antibodies directed against a peptide complementary to a particular hormone would also bind to that hormone's receptor binding site. While the ability to immunoaffinity purify a receptor for a specific hormone is a valuable technique, the concept of complementary peptide sequences binding one another is not limited to this and has also been applied to antibody–antibody interactions[8] and to peptides which bind ribonuclease S peptide.[3] These studies suggest the general applicability of this technique, and one purpose of this chapter is to consider some potential applications of the methodology. The primary intent, however, is one of a technical nature. While it may appear simple to construct complementary peptides, there are many considerations that on first examination are not obvious. In this chapter we present a composite of the technical considerations we have generated to date relative to constructing complementary peptides which will bind a given sequence.

Antiparallel and Parallel Complementary Peptides

Owing to the strides being made in peptide chemistry, it is not difficult to obtain synthetic peptides of specified sequences. Furthermore, increased interest in molecular biology has expanded the number of nucleo-

[1] K. L. Bost, E. M. Smith, and J. E. Blalock, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 1372 (1985).
[2] J. E. Blalock and K. L. Bost, *Biochem. J.* **234,** 679 (1986).
[3] Y. Shai, M. Flashner, and I. M. Chaiken, *Biochemistry* **26,** 669 (1987).
[4] K. L. Bost and J. E. Blalock, *Mol. Cell. Endocrinol.* **44,** 1 (1986).
[5] D. J. J. Carr, K. L. Bost, and J. E. Blalock, *J. Neuroimmunol.* **12,** 329 (1986).
[6] J. J. Mulchahey, J. D. Neill, L. D. Dion, K. L. Bost, and J. E. Blalock, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 9714 (1986).
[7] T. J. Gorcs, P. E. Gottschall, D. H. Coy, and A. Arimura, *Peptides (N.Y.)* **7,** 1137 (1986).
[8] L. R. Smith, K. L. Bost, and J. E. Blalock, *J. Immunol.* **138,** 7 (1987).

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tide and amino acid sequences available for study. Thus, both the sequence information and the technology are available for constructing complementary peptides; however, there are several considerations when deciding which peptide is appropriate to synthesize. Table I is an inclusive list of the possible amino acids encoded by complementary codons in both the 5′ to 3′ and 3′ to 5′ directions, wherein lies the first question. In

TABLE I
COMPLEMENTARY AMINO ACIDS

Amino acid	Codon (5′ to 3′)	Complementary codon (3′ to 5′)	Amino acid encoded by the complementary codon	
			5′ to 3′ direction	3′ to 5′ direction
Isoleucine	AUA	UAU	Tyr	Tyr
	AUC	UAG	Asp	Stop
	AUU	UAA	Asn	Stop
Methionine	AUG	UAC	His	Tyr
Leucine	CUA	GAU	Stop	Asp
	CUC	GAG	Glu	Glu
	CUG	GAC	Gln	Asp
	CUU	GAA	Lys	Glu
	UUA	AAU	Stop	Asn
	UUG	AAC	Gln	Asn
Valine	GUA	CAU	Tyr	His
	GUC	CAG	Asp	Gln
	GUG	CAC	His	His
	GUU	CAA	Asn	Gln
Phenylalanine	UUC	AAG	Glu	Lys
	UUU	AAA	Lys	Lys
Lysine	AAA	UUU	Phe	Phe
	AAG	UUC	Leu	Phe
Asparagine	AAC	UUG	Val	Leu
	AAU	UUA	Ile	Leu
Glutamine	CAA	GUU	Leu	Val
	CAG	GUC	Leu	Val
Histidine	CAC	GUG	Val	Val
	CAU	GUA	Met	Val
Glutamic acid	GAA	CUU	Phe	Leu
	GAG	CUC	Leu	Leu

TABLE I (*continued*)

Amino acid	Codon (5′ to 3′)	Complementary codon (3′ to 5′)	Amino acid encoded by the complementary codon	
			5′ to 3′ direction	3′ to 5′ direction
Aspartic acid	GAC	CUG	Val	Leu
	GAU	CUA	Ile	Leu
Tyrosine	UAC	AUG	Val	Met
	UAU	AUA	Ile	Ile
Threonine	ACA	UGU	Cys	Cys
	ACC	UGG	Gly	Trp
	ACG	UGC	Arg	Cys
	ACU	UGA	Ser	Stop
Proline	CCA	GGU	Trp	Gly
	CCC	GGG	Gly	Gly
	CCG	GGC	Arg	Gly
	CCU	GGA	Arg	Gly
Alanine	GCA	CGU	Cys	Arg
	GCC	CGG	Gly	Arg
	GCG	CGC	Arg	Arg
	GCU	CGA	Ser	Arg
Serine	UCA	AGU	Stop	Ser
	UCC	AGG	Gly	Arg
	UCG	AGC	Arg	Ser
	UCU	AGA	Arg	Arg
	AGC	UCG	Ala	Ser
	AGU	UCA	Thr	Ser
Arginine	AGA	UCU	Ser	Ser
	AGG	UCC	Pro	Ser
	CGA	GCU	Ser	Ala
	CGC	GCG	Ala	Ala
	CGG	GCC	Pro	Ala
	CGU	GCA	Thr	Ala
Glycine	GGA	CCU	Ser	Pro
	GGC	CCG	Ala	Pro
	GGG	CCC	Pro	Pro
	GGU	CCA	Thr	Pro
Cysteine	UGC	ACG	Ala	Thr
	UGU	ACA	Thr	Thr
Tryptophan	UGG	ACC	Pro	Thr

which direction should a complementary peptide be encoded? Initially, the usual 5′ to 3′ translational direction would seem to be the only logical possibility. However, since the middle base of the codon specifies the hydropathic nature of the amino acid,[2] peptides encoded in either direction using the same RNA sequence and the same reading frame will be similar with respect to their hydropathicity. Stated simply, the middle base is always the second base regardless of the direction of reading. Since the middle base determines the hydropathic nature of an amino acid, one would predict that peptides encoded in the 5′ to 3′ or 3′ to 5′ direction might have similar binding characteristics if a similar hydropathic nature was important. In one case studied,[2] we found that peptides encoded by the RNA complementary to the mRNA for ACTH in either the 5′ to 3′ or 3′ to 5′ direction had almost identical abilities to bind ^{125}I-ACTH. In this particular instance, ^{125}I-ACTH binding to the antiparallel or parallel encoded peptides was similar; however, this may not always be the case.

A second reason to consider 3′ to 5′ encoded complementary peptides is the finding that regions of complementarity between known receptor–ligand pairs were found to occur in the 3′ to 5′ direction.[9] One region of complementarity between interleukin 2 and its receptor was subsequently shown to represent a binding-site sequence for this receptor–ligand pair.[10] While the biological mechanisms by which these 3′ to 5′ complementarities occur in nature is unclear at present, additional sequence information on pairs of interacting proteins will be necessary to understand this occurrence.

In conclusion, when synthesizing complementary peptides based on nucleotide sequences, consideration should be given to peptides encoded in both the 5′ to 3′ and 3′ to 5′ directions. Presently, adequate experimental evidence is not available to determine which peptide will be the most appropriate for each application of the methodology.

Consensus Sequences

It is possible to generate a complementary peptide from a primary amino acid sequence without prior knowledge of the nucleotide sequence. This process is analogous to constructing multiple oligonucleotide probes from primary sequence information, but it is more restricted in the sense that a single amino acid (e.g., glutamine) encoded by two different codons

[9] K. L. Bost, E. M. Smith, and J. E. Blalock, *Biochem. Biophys. Res. Commun.* **128**, 1373 (1985).

[10] D. A. Weigent, P. D. Hoeprich, K. L. Bost, T. K. Brunck, W. E. Reiher, and J. E. Blalock, *Biochem. Biophys. Res. Commun.* **139**, 367 (1986).

TABLE II
AMINO ACID SEQUENCES FOR SUBSTANCE P AND ITS 3′ TO 5′ COMPLEMENTARY PEPTIDE

Substance P amino acid sequence[11]										
1	2	3	4	5	6	7	8	9	10	11
Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met
3′ to 5′ possible amino acid complements (see Table I)										
1	2	3	4	5	6	7	8	9	10	11
Ser	Gly	Phe	Gly	Val	Val	Lys	Lys	Pro	Asp	Tyr
Ser	Gly	Phe	Gly	Val	Val	Lys	Lys	Pro	Glu	
Ala	Gly		Gly					Pro	Asp	
Ala	Gly		Gly					Pro	Glu	
Ala									Asn	
Ala									Asn	
Consensus complementary sequence based on 3′ to 5′ possibilities										
1	2	3	4	5	6	7	8	9	10	11
Ala	Gly	Phe	Gly	Val	Val	Lys	Lys	Pro	Asn	Tyr
Actual complementary sequence based on nucleotide sequence read 3′ to 5′										
1	2	3	4	5	6	7	8	9	10	11
Ala	Gly	Phe	Gly	Val	Val	Lys	Lys	Pro	Asn	Tyr

(e.g., CAA and CAG) is complemented by only one amino acid (e.g., leucine in the 5′ to 3′ direction and valine in the 3′ to 5′ direction) (see Table I).

An example of constructing a complementary peptide directly from primary sequence information is given in Table II. From the published amino acid sequence for substance P,[11] all the possible 3′ to 5′ complementary amino acids were listed from Table I. Obviously, only the amino acids at positions 1 and 10 are questionable as to the complementary amino acid which should be used, and, in fact, only six peptides would have to be synthesized to cover all the possible combinations. However, several educated guesses can be made to further restrict the amino acids that should be used. First, depending on the species one is working with, preferred codon usage tables[12] can be utilized to determine the probability of which particular codon would be used for each amino acid for, in this example, substance P. The corresponding complementary codons could then be weighted appropriately. Second, the frequency of occurrence of a particular complementary amino acid at a single position can also be taken into account. For example, at position 1 it is likely that alanine would be the appropriate choice as a complement since it occurs four out

[11] M. M. Chang and S. E. Leeman, *J. Biol. Chem.* **245**, 4784 (1970).
[12] R. Grantham, C. Gautier, and M. Gouy, *Nucleic Acids Res.* **8**, 1893 (1980).

of six times. Third, it can also be appropriate to use the set of possible complementary amino acids encoded in both the 5′ to 3′ and 3′ to 5′ directions to select the most likely amino acid. Once again, this can be done without dramatically changing the hydropathicity at a single position since the middle base specifies the hydropathic nature of an amino acid.

As an example, it is not clear in Table II which complementary amino acid should occupy position 10. If one considers the possible complementary amino acids in the 5′ to 3′ direction for leucine given in Table I (i.e., Glu, Gln, Lys, Gln) with the possible 3′ to 5′ complementary amino acid (i.e., Asp, Glu, Asp, Glu, Asn, Asn), an amine would be the most likely choice based simply on the number of amines versus the number of acids. Therefore, the consensus complementary peptide for substance P would have alanine in position 1 and asparagine in position 10, using the rationale described above. When compared with the complementary peptide sequence generated by reading the nucleotide sequence complementary to the nucleotide sequence for substance P in the 3′ to 5′ direction, the consensus sequence is identical (see Table II).

It should be pointed out that the same rationale can be used to generate consensus complementary peptides using 5′ to 3′ encoded complementary amino acids. However, the number of possibilities increases in this direction when compared to the possible complementary amino acids in the 3′ to 5′ direction. For example, alanine is complemented by cysteine, glycine, arginine, or serine in the 5′ to 3′ direction but only by arginine in the 3′ to 5′ direction. The restricted nature of the set of 3′ to 5′ complementary amino acids results from the first base of the sense codon complementing the first base of the antisense codon. In the 5′ to 3′ direction, the third base of the sense codon complements the first base of the antisense codon. Owing to the degeneracy of the genetic code, 5′ to 3′ complementary amino acids are less restricted.

Experimentally, two peptides were synthesized based on the consensus complementary sequence for substance P. One peptide had asparagine at position 10, which also corresponds to the nucleotide-derived complement (see Table II), and the second peptide had glutamic acid at position 10. While either peptide coated onto microtiter wells was capable of binding tritiated substance P, the glutamic acid-containing peptide routinely bound more radiolabel than the asparagine-containing peptide. Figure 1 shows the ability of the glutamic acid-containing complementary peptide to bind radiolabel and the ability of this binding to be blocked by unlabeled substance P or the analog [D-Pro^2-D-Phe^7-D-Trp^9]substance P. Thus, here is another example of a 3′ to 5′ encoded complementary peptide binding its radiolabeled ligand.

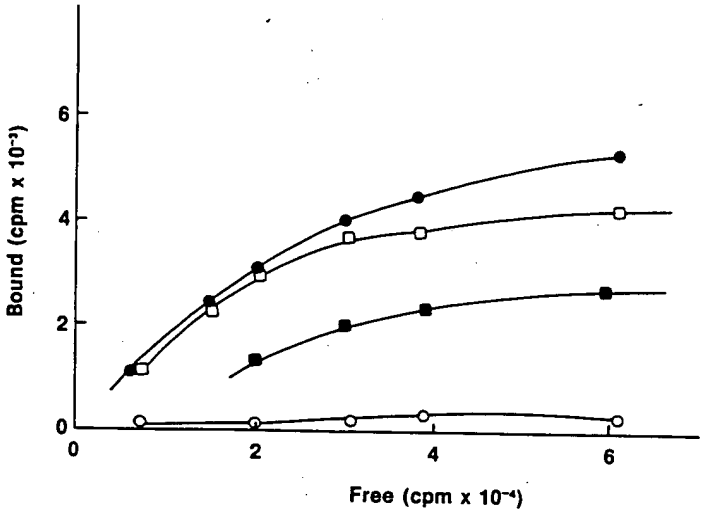

FIG. 1. Binding of tritiated substance P to its 3′ to 5′ complementary peptide. The peptide NH_2-Tyr-Glu-Pro-Lys-Lys-Val-Val-Gly-Phe-Gly-Ala-COOH was placed in carbonate buffer (pH 8.6) at 0.5 mg/ml and coated onto microtiter wells (0.1 ml/well) overnight at 4°. After blocking within 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hr, varying concentrations of ^{3}H-labeled substance P were added in PBS containing 0.5% BSA and 0.02% Tween 20 for 2 hr. The amount of ^{3}H-labeled substance P was determined in the presence of 0.5% BSA (●), a 500-fold excess of soluble complementary peptide (□), a 500-fold excess of soluble complementary peptide which had been cross-linked to BSA via glutaraldehyde (■), or a 500-fold excess unlabeled substance P (○). The ability of a 500-fold excess of the analog [D-Pro^2-D-Phe^7-D-Trp^9]substance P to block binding was indistinguishable from that of unlabeled substance P.

Problematic Sequences: Stop Codons

Certain codon sequences can present problems in attempts to construct complementary peptides. For example, the 5′ to 3′ complementary codon to the UUA codon for leucine is UAA which is a stop codon. If the complementary peptide to be synthesized is of sufficiently small size that terminating the peptide and starting a second one are not appropriate, an amino acid can be substituted for the stop codon. Routinely, we select this substitution using rules similar to those for generating consensus complementary peptides. For example, in the 5′ to 3′ direction, leucine is complemented by the amino acids Glu, Gln, Lys, and Gln. In the 3′ to 5′ direction, the codon UUA is complemented by AAU encoding Asn. Thus, on a frequency basis, this should be an amine, and two possibilities for substituting for this UAA stop codon would be Gln or Asn.

Solid-Phase Binding Assays Using Complementary Peptides

Choice of Complementary Peptide

In addition to the considerations discussed above relative to selecting an appropriate complementary peptide to synthesize, there are several additional factors that should be noted when conducting solid-phase binding assays. First, the length of the complementary peptide generally directly influences the affinity of binding to a particular ligand. For example, the complementary peptide to ACTH 1–24 bound this hormone with a K_D of 0.3 nM,[2] whereas smaller pairs of complementary peptides had dissociation constants in the micromolar range (see Table III). Often it is difficult to demonstrate specific binding using very small peptides (e.g., hexamers), probably due to limited secondary structure in solution and to the restrictions imparted to a small peptide bound to a solid support. Second, peptides with opposite amino to carboxy orientations have been shown to have similar[3] or differing (K. L. Bost and J. E. Blalock, unpublished observations) abilities to bind a ligand. The significance of this observation is presently under investigation. Third, especially with short peptides, the amount of nonspecific binding can sometimes be reduced by neutralizing the dipole moment of the complementary peptide. This can be accomplished by amidating carboxy termini and acetylating amino termini to reduce charge–charge interactions.

TABLE III
DISSOCIATION CONSTANTS FOR PAIRS OF COMPLEMENTARY PEPTIDES

Complementary peptide pair	Number of amino acids	Dissociation constant (M)	Ref.
ACTH 1–24/5′ to 3′ complementary peptide	24	0.3×10^{-9}	2
ACTH 1–24/3′ to 5′ complementary peptide	24	0.3×10^{-9}	2
γ-Endorphin/5′ to 3′ complementary peptide	17	2×10^{-5}	5
LHRH/5′ to 3′ complementary peptide	10	$\sim 1 \times 10^{-4}$	6[a]
Ribonuclease S peptide/5′ to 3′ complementary peptide	20	1.3×10^{-6}	3
Ribonuclease S peptide/5′ to 3′ inverted complementary peptide	20	1.2×10^{-6}	3
Substance P/3′ to 5′ complementary peptide	9	6×10^{-6}	[b]

[a] The dissociation constant was estimated from the concentration of peptide necessary to cause a 50% inhibition of a biological assay.
[b] K. L. Bost and J. E. Blalock, unpublished observations.

Solid-Phase Binding Assays

The reader is referred to other publications[13,14] which describe general aspects of solid-phase binding assays. We typically coat microtiter wells with 10–50 μg of the peptide in an appropriate buffer overnight at 4°. At this point, wells are washed and blocked with an appropriate irrelevant protein [e.g., 1% bovine serum albumin (BSA) for 2 hr]. Reaction times with radiolabeled ligands are typically 2 hr in buffer such as phosphate-buffered saline (PBS) with 0.5% BSA and 0.05% Tween 20 after which unbound radiolabel is washed out with a similar buffer.

There are several variables in solid-phase binding assays using microtiter plates which should be optimized for each system. The first variable is the microtiter plates which are used as a solid support. Polyvinyl plates have been used extensively because of their high protein-binding capacity. Recently, however, high-binding polystyrene plates like Nunc-immuno plates (Interlab, Newbury Park, CA) or Immulon plates (Dynatech, Chantilly, VA) have become available, and these are routinely used in our laboratory. These polystyrene plates have uniform binding characteristics which minimize well-to-well variations. A second variable is the method used to coat peptides to wells. Surprisingly, this is a very important variable which becomes more critical as the size of the peptide to be coated is decreased. There is no reason to assume that all the orientations which a peptide assumes on adsorbing to a solid support will be conducive for binding a radiolabeled ligand. Therefore, we routinely determine optimal methods for coating a particular peptide prior to performing binding assays. Typically, proteins are coated in carbonate buffer, around pH 9.0[1] or in phosphate-buffered saline, pH about 7.0[2]; however, this is not always optimal for peptides. Activating plates by pretreatment with 20 m*M* glutaraldehyde for 1 hr, followed by two 0.15 *M* NaCl washes and subsequent addition of the peptide for coating, can be an effective alternative.[15] Conversely, microtiter plates are commercially available (Micro Membranes, Inc., Newark, NJ) which allow covalent coupling of proteins or peptides to the surface. As a final consideration, the binding ability of some peptides can be markedly enhanced by coupling to a carrier protein (see the following section).

Nonspecific binding of the radiolabeled ligand can usually be controlled by the number of washing steps and by the composition of the

[13] D. N. Orth, this series, Vol. 37, p. 22.
[14] G. E. Trivers, C. C. Harris, C. Rougeot, and F. Dray, this volume [40].
[15] L. M. Kuo and R. J. Robb, *J. Immunol.* **137**, 1538 (1986).

washing buffer. An optimal washing buffer should permit specific binding (i.e., binding which is blockable by unlabeled ligand and an appropriate antagonist) to occur but should minimize nonspecific binding. We typically use phosphate-buffered saline with 0.5% BSA and 0.05% Tween 20; however, depending on the assay, ovalbumin, bacitracin, and 1% fetal calf serum have been used alone or in combination to give optimal binding results.

An alternative method to using microtiter plates is that described by Shai *et al.*[3] Here peptides are immobilized on silica beads, and binding interactions are analyzed using high-performance affinity chromatography. The advantages of using this method over solid-phase binding assays using microtiter plates has been previously reviewed.[16–18]

Solid-Phase versus Solution Binding

One final observation relative to solid-phase binding assays should be made. Earlier, reference was made to the ability of ^{3}H-labeled substance P to bind microtiter wells previously coated with its complementary peptide. This binding could be blocked by unlabeled substance P or the antagonist [D-Pro^2-D-Phe^7-D-Trp^9]substance P but could not be effectively blocked with a 500-fold excess of soluble complementary peptide (see Fig. 1). On coupling the glutamic acid-containing complementary peptide for substance P to BSA, this conjugate was able to effectively block binding (~50%), whereas BSA was without effect. Thus, peptides or proteins interacting with a third surface may not have similar binding properties when placed in solution. Therefore, if binding of complementary peptide pairs in solution is weak, one should consider coupling a peptide to a solid support or a carrier protein. Furthermore, the binding ability of some peptides may be enhanced by coupling to a carrier protein prior to immobilization on a solid support.

Use of Complementary Peptides

Antireceptor Antibodies

Polyclonal antibodies against the ACTH, endorphin, and LHRH receptor binding sites have been produced in rabbits by immunization with the appropriate complementary peptides.[1,4–7] The ability to generate antireceptor antibodies for all three hormone receptors supports the fidelity of

[16] H. E. Swaisgood and I. M. Chaiken, *J. Chromatogr.* **327,** 193 (1985).
[17] H. E. Swaisgood and I. M. Chaiken, *Biochemistry* **25,** 4148 (1986).
[18] I. M. Chaiken, *J. Chromatogr.* **376,** 11 (1986).

this methodology. Before discussing the technical aspects, there are some general considerations which need to be addressed. First, the responses of individual rabbits to a particular antigen is quite heterogeneous; thus, some rabbits produce high titers whereas others do not. It would be advisable to immunize multiple animals and screen them, using immunoassays,[13,14] for the best producer. Second, producing antireceptor antibodies results in physiological responses in the immunized animal[7] (K. L. Bost and J. E. Blalock, unpublished observations). In other words, animals are making anti-self antibodies. It is important to realize that these anti-self antibody responses may not only be relatively low-titer responses but also may be transient. Thus, it is necessary to screen each bleed to determine the highest antireceptor titer.

Previous publications[19,20] have dealt with antibody production from a technical point of view. Since most peptides are not very immunogenic, coupling to a carrier protein is necessary prior to immunization. While there are a large variety of commercially available cross-linking agents (Pierce Chemical, Rockford, IL), the choice of cross-linker can affect the antireceptor antibody titer. Table IV shows that mice immunized with keyhole limpet hemocyanin (KLH) conjugated to the 5′ to 3′ complementary peptide for ACTH 1–24 (HTCA) via glutaraldehyde[1] routinely gave higher titers than carbodiimide coupling,[10] but not necessarily higher antireceptor antibody titers. In addition to pointing out that the method for cross-linking a peptide to a carrier protein can affect the antibody response, these results also show that antipeptide and antireceptor titers do not always correlate. In other words, only a portion of the anticomplementary peptide antibodies produced should be expected to cross-react with the receptor binding site. For this reason, it is important to screen sera not only against the immunogen but also against the receptor. Furthermore, biological assays to determine the agonistic or antagonistic properties of the antibodies are extremely helpful. For example, antibodies against HTCA were assayed for their ability to bind Y-1 adrenal cells[1] and for their ability to induce rounding and steroidogenesis of these ACTH receptor-positive cells.[1]

Purification of antireceptor antibodies is most easily accomplished by removing antibodies against the carrier protein[1] or by affinity purification using peptide-conjugated affinity columns. Peptides are routinely conjugated to Affi-Gel 10 or Affi-Gel 15 (see Technical Bulletin 1099, Bio-Rad, Richmond, CA), and this peptide-conjugated affinity column is then used

[19] B. A. L. Hurn and S. M. Chantler, this series, Vol. 70, p. 104.
[20] G. Galfre and C. Milstein, this series, Vol. 73, p. 1.

TABLE IV
ANTIBODY PRODUCTION TO THE 5′ TO 3′ COMPLEMENTARY PEPTIDE FOR ACTH 1–24 (HTCA) IN BALB/c MICE

Mouse[a]	Coupling method[b]	Adjuvant[c]	Antibody titer^{-1}	
			Anti-HTCA[d]	Anti-ACTH receptor[e]
1	Glutaraldehyde	Freund's	4800	1000
2	Glutaraldehyde	Ribi	6400	2000
3	Carbodiimide	Freund's	2400	800
4	Carbodiimide	Ribi	2400	1600

[a] Three mice were injected per group, and representative mice from each group are shown here. Mice were injected twice with 100 μg of peptide with 10 days between injections. Blood was taken for antibody screening 10 days after the final immunization.
[b] HTCA was coupled to keyhole limpet hemocyanin (KLH) with glutaraldehyde[1] or carbodiimide[10] as previously described. Coupling efficiency for glutaraldehyde was approximately 60%, whereas for carbodiimide it was approximately 15%. The amount of peptide each mouse received was adjusted accordingly.
[c] Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, MO) or Ribi MPL + TDM adjuvant (Ribi Immunochem Research, Inc., Hamilton, MT) was used.
[d] Antibody titers were determined against HTCA coated onto microtiter plates.
[e] Antibody titers were determined against Y-1 adrenal cells fixed to microtiter plates as previously described.[1]

to specifically purify the antibodies. The advantages of using Affi-Gel 10 or 15 include rapid, highly efficient coupling plus the presence of a spacer arm to prevent steric hindrance. Purified antireceptor antibodies can then be used for immunoaffinity purification of receptors from the surface of receptor-positive cells.[21,22] Solubilization of receptors from cells, which would be necessary prior to immunoaffinity procedures, has been dealt with extensively.[23,24]

Antigen–Antibody Interactions

Previously, we have shown that antibodies made against pairs of complementary peptides have an idiotype–antiidiotype relationship.[8] As judged by radioimmunoassay, antibodies against ACTH could bind antibodies against its 5′ to 3′ encoded complementary peptide, HTCA, and this binding could be blocked with either ACTH or HTCA. Furthermore,

[21] M. Wilchek, T. Miron, and J. Kohn, this series, Vol. 104, p. 3.
[22] G. J. Calton, this series, Vol. 104, p. 381.
[23] L. M. Hjelmeland and A. Chrambach, this series, Vol. 104, p. 305.
[24] J. V. Renswoude and C. Kempf, this series, Vol. 104, p. 329.

antibodies directed against β-endorphin could bind antibodies against the 5′ to 3′ encoded complementary peptide for γ-endorphin. In each case, the antibodies were binding one another at or near their antigen binding sites as evidenced by the ability of the appropriate ligand to block binding. By definition, these antibodies had an idiotype–antiidiotype relationship. Thus, this method allows one to generate antiidiotypic antibodies in a predetermined rather than a random manner. Whether HTCA sequences or their analogs can be found within the antigen combining sites of anti-ACTH antibodies and vice versa is presently under investigation. In the one case studied to date, however, we have found that complementary sequences located within the hypervariable region (i.e., antigen binding region) of a monoclonal antibody and its protein antigen specify the contact points for these interacting proteins (K. L. Bost and J. E. Blalock, unpublished observations). Thus, it may be that all the possible antigens or their peptide equivalents to which an individual can respond are encoded in the nucleic acids complementary to those encoding antibody binding sites.

Future Directions

There are many potential situations in which complementary peptides can be applied, and future studies will determine the uses. Initial studies suggest that the complementary peptide to ACTH, when injected *in vivo*, can partially inhibit stress-induced steroid production by binding ACTH (K. L. Bost and J. E. Blalock, unpublished observations). It may also be possible to use such binding peptides as substitutes for antibodies in radioimmunoassays or enzyme-linked immunosorbent assays. Along a more theoretical vein, it may be possible to identify peptide equivalents of nonpeptide ligands. If binding sites for these nonpeptide ligands could be identified from receptors or specific antibodies, then sequences complementary to these binding sites may represent peptide analogs similar to the morphinelike peptide Tyr-Gly-Gly-Phe.

Molecular Biomethods Handbook

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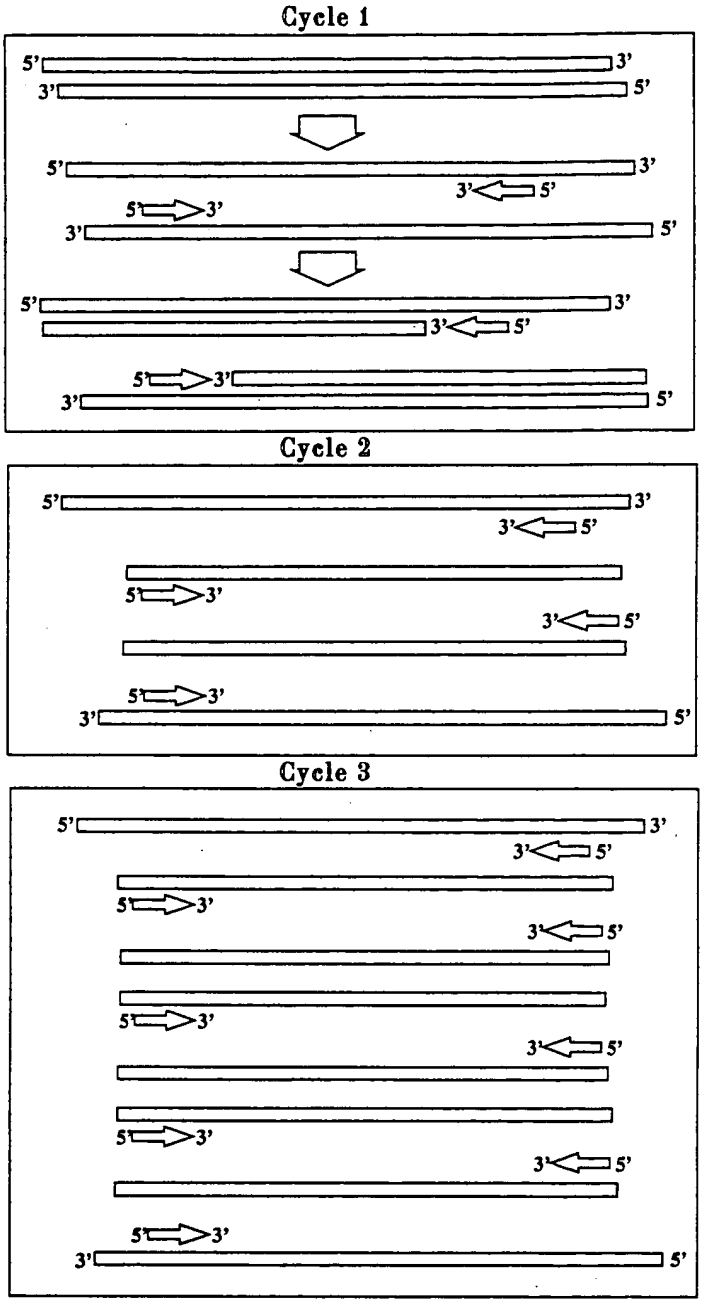

Fig. 2. First few cycles in the PCR.

2.1. Consideration and Design of Primers

Primers used in the PCR are generally designed using information based on existing sequences of close similarities or evolutionary conserved sequences found by searching genetic databases, such as Genbank/EMBL. Amino acid sequence information may also be used to provide deduced nucleotide sequences from which primers may be

designed. In general, the primers should have a matched GC content of approx 50% and must not have the potential to form primer–dimer structures or be self complementary, both of which adversely affect the PCR *(3)*.

A modification of the PCR, termed anchored PCR, has been developed for cases in which either the 3' or 5' portion of the sequence under study is not known. It is possible when amplifying cDNA produced from eukaryotic mRNA to make use of the characteristic polyA tail found at the 3' end. In this case, a polyA primer binding to the polydT in the cDNA acts as one primer leaving only one flanking primer to be designed. A similar effect may be obtained by ligating a linker sequence to the end of the cDNA, such as poly dG; a poly dC primer may be used in this case (linker-PCR). Finally, where sequence information is limited it is possible to use gene families and sequences of close similarity to design degenerate primers having either a base analog such as inosine at a particular position within the primer, or by having alternative bases at that position *(4)*. These primers require a degree of optimization of the annealing conditions since they may not directly match the sequence they are designed to bind, but are very useful where sequence information is limited. The choice of annealing temperature is critical in most PCRs since if a temperature is chosen that is very close to the melting temperatures of the primers no mismatches are likely to be tolerated. However, in some cases it may be desirable to lower the annealing temperature to allow the reaction to proceed even if there are one or two mismatches. The design of primers is critical not only for specific amplification but also to allow further post-PCR manipulations through the inclusion of restriction sites or promoter sites in primers. A number of computer programs are currently available to aid in the process of primer design and optimization of annealing temperatures. It is also possible to quantify the PCR in certain circumstances by amplification of an internal standard. This usually consists of the simultaneous amplification from a standard template in addition to the PCR being undertaken. This is especially useful for the determining the extent of viral or bacterial infections or in gene expression studies *(5)*.

2.2. Thermostable DNA Polymerases

During the initial development of the PCR the enzyme used to carry out the extension step was usually the Klenow fragment of DNA polymerase I. However, because this is heat labile, fresh enzyme was required during each cycle since the high denaturation temperatures denatured not only the template DNA but also the enzyme. This made the technique labor-intensive and quite costly. The introduction of thermostable DNA polymerases into the PCR transformed the technique and allowed full automation since only one aliquot of an enzyme needed to be added at the start of the reaction.

2.3. Taq DNA Polymerases

The first and most commonly employed thermostable DNA polymerase was that isolated from a bacterium *T. aquaticus* found in the hot springs of Yellowstone National Park. Taq and its recombinant form Amplitaq® both have relatively high processivity, a 5'–3' exonuclease activity, and a temperature optimum of 72°C *(6)*. The polymerase does lack 3'–5' proofreading exonuclease activity and does appear to contribute to misincorporation of nucleotides. A further derivative of these enzymes is the Stoffel fragment, which has a higher thermostability and is less sensitive to changes in Mg^{2+}

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